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High Pressure Enhances the Growth Rate of the Thermophilic Archaebacterium *Methanococcus thermolithotrophicus* without Extending Its Temperature Range

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Temperature and hydrostatic pressure are essential in determining the assemblage of species in their specific biotopes. To evaluate the effect of high pressure on the range of viability of thermophiles, the pressure and temperature dependence of the growth of the methanogenic archaebacterium *Methanococcus thermolithotrophicus* was investigated. High pressure up to 50 MPa enhanced the growth rate without extending the temperature range of viability. The optimum temperature remained unaltered (65°C). Beyond 50 MPa, cell lysis predominated over cell proliferation. Destabilization was also observed at temperatures below and above the optimum growth temperature (<60°C, ≥70°C) and at low substrate concentrations.

The ecology of marine biotopes and the distribution pattern of organisms on the surface of the earth are determined by temperature, pressure, and water activity (6). Elevated hydrostatic pressure gains importance at two levels: first, on the ocean floor where pressure may reach 120 MPa and, second, in hydrothermal vents where pressure keeps water in its liquid state at temperatures beyond its normal boiling point. Hydrothermal degradation reactions of biological macromolecules under these extreme conditions prove that the presence of water in its liquid state cannot be a sufficient criterion for the occurrence of life. Previous studies clearly suggested that, provided nature has not furnished an entirely new repertoire of biomolecules to cope with extremes of temperature and pressure, the limits of viability with regard to temperature cannot be far beyond 110°C (3, 7, 12). Reports on "Black smoker" bacteria growing at >250°C and 26.5 MPa (1) were refuted by Trent et al. (11). In this context one may ask whether increased pressure can extend the range of viability due to stabilizing effects reported for a number of enzymes (for a review, see reference 6).

Quantitative evaluation of the growth of hyperthermophilic microorganisms is exceedingly difficult at high pressure. Therefore, we investigated a moderate thermophile in which alterations in the optimum growth temperature could be measured with sufficient accuracy. Previous experiments indicated that elevated pressure up to 45 MPa was not sufficient to shift the temperature limit of growth and reproduction of *Bacillus stearothermophilus* to higher values. On the contrary, the upper temperature of colony formation was shown to be decreased from 70 to 67°C, whereas at ≈56 MPa complete growth inhibition was observed (13).

Methanococcus thermolithotrophicus, a methanogenic archaebacterium with a temperature range of growth from 30 to 70°C (5), is phylogenetically closely related to hyperthermophilic bacteria. It gains its metabolic energy based on the reaction

$$4H_2 + CO_2 \rightleftharpoons CH_4 + 2H_2O \quad (1)$$

The gaseous substrates suggest that the reaction volume of the reaction is negative, so that increased pressure is expected to favor product formation, i.e., bacterial growth.

MATERIALS AND METHODS

Incubation of a 5% bacterial suspension in 20 ml of medium was performed at 65°C in serum flasks containing a gas mixture of 80% H_2 and 20% CO_2 at 0.2 MPa. To provide constant pH conditions at high pressure and temperature, standard minimal medium was supplemented with 120 mM HEPES (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (G. Bernhardt, Ph.D. thesis, University of Regensburg, 1986). To minimize H_2 leakage and to optimize reproducibility at high pressure and high temperature, nickel tubes were used in a set of autoclaves connected in series (2). Pressure was transduced and monitored as described previously (9). Quantification of bacterial growth made use of cell counting in a Neubauer chamber (0.05 by 0.05 by 0.02 mm). For electron microscopy (JEOL Jem 100 C electron microscope), cells were fixed in 2.5% (wt/vol) glutaraldehyde and shadowed with platinum-iridium.

RESULTS AND DISCUSSION

M. thermolithotrophicus around normal atmospheric pressure has its optimum growth temperature at 65°C (5); the final cell density in the stationary phase amounts to 2×10^8 cells per ml. At elevated pressure, the growth of the bacterium was enhanced significantly. Beyond 50 MPa, lysis became predominant; at 100 MPa, growth enhancement and lysis compensated for each other so that cell proliferation vanished after an initial burst (Fig. 1B). At 70°C growth was inhibited; at moderate pressure (20 MPa), the increase in cell number did not exceed one single cell cycle (Fig. 1C). The bimodal growth profile observed at 56°C and elevated pressure was repeatedly confirmed, also after reincubation of the bacterium in fresh medium (Fig. 1A). Similar growth characteristics were observed after the cells were incubated at 56°C and 22 MPa and then stored at ≈5°C for 2 days, whereas preincubation at 56°C and 22 MPa for 24 h (omitting the intermediate cooling period) resulted in a sigmoidal growth curve (Fig. 1A). Whether the incubation mixture contained variants differing in barotolerance (4) or whether

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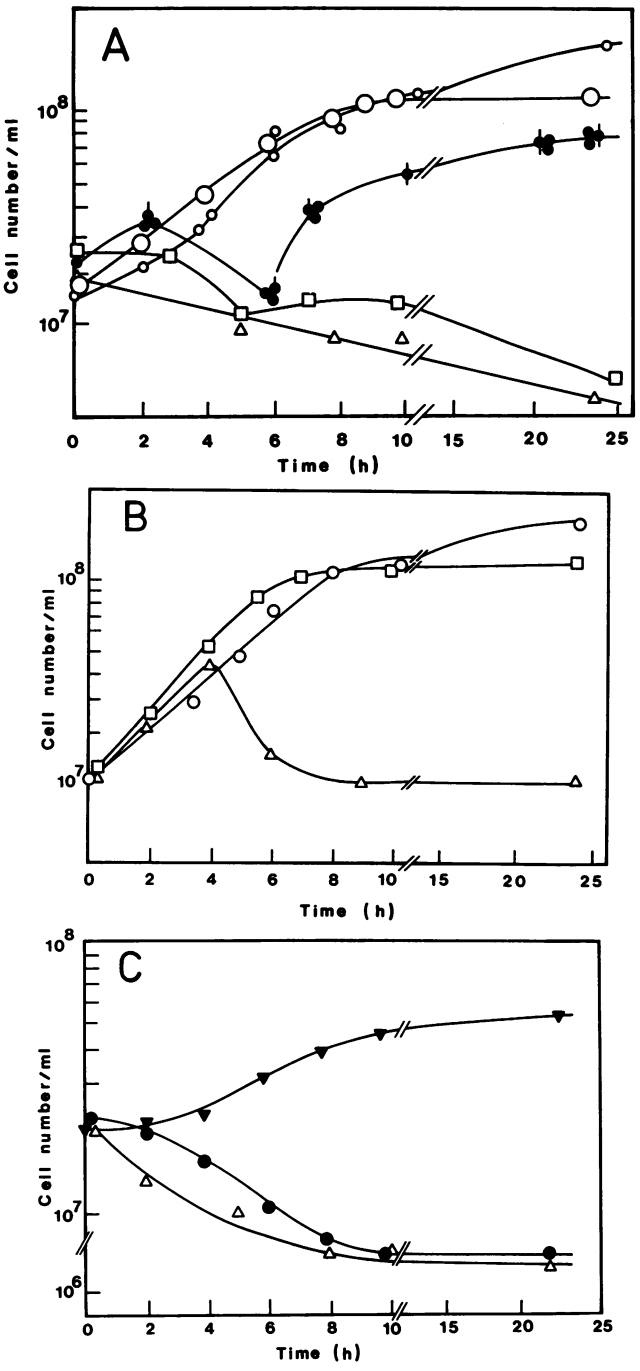

FIG. 1. Pressure effect on the growth of *M. thermolithotrophicus* in minimal medium (pH 6.9) in the presence of 120 mM HEPES (7). (A) Growth curves at 56°C and varied pressure. Pressure of 22 MPa (●, ○, and ◐), 50 MPa (□), or 100 MPa (△) was applied after 10 h of preincubation at normal atmospheric pressure. Symbols: (○) reference at 0.1 MPa; (●) samples preincubated at 0.1 MPa for 10 h and transferred into fresh medium (subsequent incubation at 22 MPa led to a bimodal growth curve); (○) cells preincubated at 22 MPa for 24 h and then transferred into fresh medium (further incubation at 22 MPa resulted in a growth curve similar to that observed under normal atmospheric pressure); (◐) after cells reached stationary growth at 22 MPa, they were stored under anaerobic conditions for 48 h at 5°C and normal atmospheric pressure; (●) reincubation at 22 MPa led back to the initial biphasic profile. (B) Growth curves at 65°C at 0.1 MPa (○), 50 MPa (□), or 100 MPa (△). (C) Growth curves at 70°C at 20 MPa (▼) and at 75°C and 20 MPa (●) or 100 MPa (△).

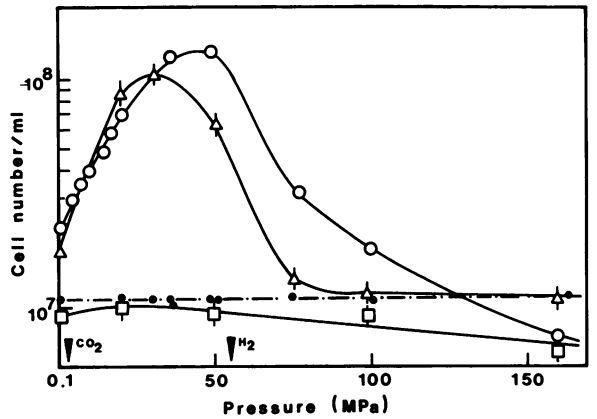

FIG. 2. Effect of temperature and pressure on the growth of *M. thermolithotrophicus* in 3 ml of minimal medium (pH 6.9) in the presence of 120 mM HEPES plus 7 ml of H_2-CO_2 at 0.4 MPa. Growth was determined after 10 h at 65°C (○), 70°C (△), and 75°C (□). Arrows indicate the pressures at which the total CO_2 and H_2 volumes were dissolved completely in the medium; (●) initial cell number of the inoculum.

the bacterium underwent pressure adaptation cannot be answered.

Figure 2 illustrates the effect of pressure on the stationary net growth at various temperatures. Increased hydrostatic pressure did not shift the optimum growth temperature to higher values. Instead, the cells became unstable, even at 70°C where under normal conditions the doubling time is still close to its optimum value (Table 1).

In these experiments, it was essential that cell proliferation was not restricted by insufficient nutrient concentration. As indicated by experiments at various substrate concentrations, altering the inoculum/substrate ratio by a factor of 4 did not alter the growth characteristics (Fig. 1A). The arrows in Fig. 2 indicate the pressures at which CO_2 and H_2 were fully dissolved in the culture medium. At low hydrostatic pressure and high temperature (≈3 MPa and 70°C), the H_2 concentration was on the order of 20 mM, exceeding the concentration in natural biotopes by at least 4 orders of magnitude (10).

Investigation of the stability and morphology of the cells under various growth conditions showed that pressure, temperature, and substrate concentration played an essential role in determining the size, shape, and stability of *M. thermolithotrophicus*. In the absence of substrate (CO_2 and H_2), the cell number remained constant over >70 h; the cells were morphologically normal and started dividing as soon as substrates were added as carbon sources. During the inter-

TABLE 1. Effect of pressure and temperature on the doubling time of *M. thermolithotrophicus*

Temp (°C)	Doubling time (h)[a]		
	0.1 MPa	20 MPa	50 MPa
56	1.9 (1.1)[b]	2.6	Lysis
65	2.0 (0.9)[b]	1.9	1.2
70	5 ± 1 (1.3)[b]	6.9	Lysis

[a] Incubation was in nickel tubes as described previously (2), determination was during the initial logarithmic phase, and the estimated error was 10%.
[b] Incubation in 100-ml serum flasks under shaking; determination was during the exponential phase (5).

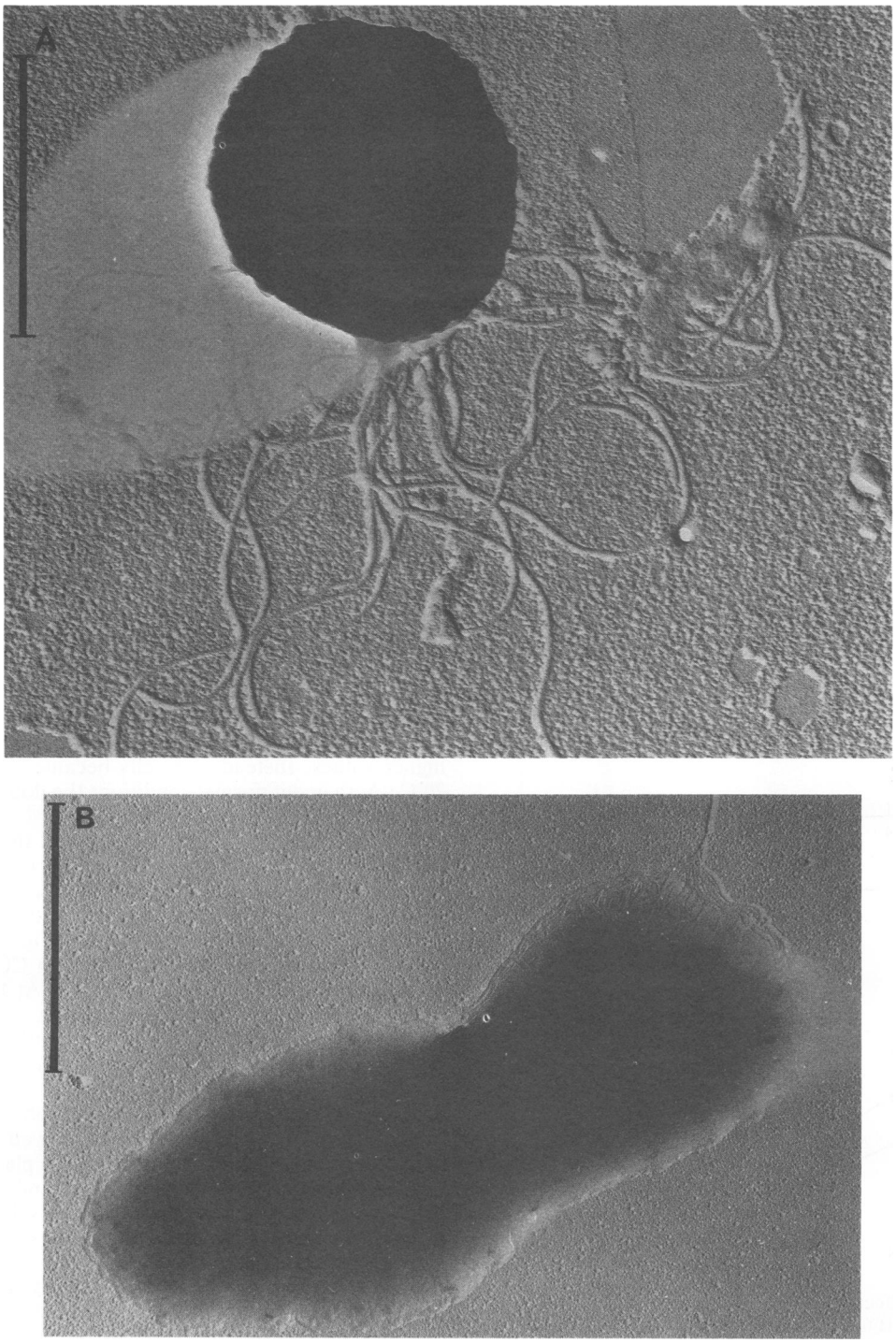

FIG. 3. Electron micrographs of *M. thermolithotrophicus* (A) under standard conditions and (B) after 8 h at 65°C and 100 MPa. For shadowing, the cells were fixed on collodium-coated grids and exposed to Pt-Ir at an angle of 7°. Bars, 1 μm.

mediary decrease in cell number at 56°C and 22 MPa, the cells showed anomalous instability with respect to mechanical stress as well as oxygen. The same held under conditions of insufficient nutrient supply (3 ml of medium plus 7 ml of H_2-CO_2 at 0.1 MPa). [It should be noticed that *Methanobacterium wolfei* under hydrogen deficiency exhibits similar behavior (8)]. The rupture of cells was accompanied by a complete decay in fluorescence emission. Minicells formed under these conditions, and also at high temperature (≥70°C) and pressures beyond 140 MPa, still showed fluorescence emission. They may represent membrane vesicles rather than complete cells.

Under high pressure, distinct morphological changes occurred. They went far beyond the wide range of variability commonly observed for archaebacteria. Whereas normal dividing cells at atmospheric pressure are spherical, with a diameter of ≈1 μm, elevated pressure (≥70 MPa at 65°C) led to anomalousy large, elongated cells which were obviously

perturbed with respect to cell division (Fig. 3). This observation corroborates earlier findings for *Escherichia coli*, in which a pressure of 45 MPa causes filamentous growth at a slow rate (14).

In summary, high hydrostatic pressure up to ca. 50 MPa enhanced the growth rate without extending the temperature range of viability. Considering the metabolic equation for methanogenic bacteria, at moderate pressure the negative sign of the activation volume predominated. The unchanged yield indicates that the reaction volume of the overall reaction does not play a significant role. This is not surprising, since the gaseous substrates in reaction (1) are dissolved completely and react in the liquid state. At high pressure and high temperature, *M. thermolithotrophicus* undergoes lysis due to "metabolic dislocation" and protein denaturation (6). Pressure adaptation is only observed at low temperature (56°C). Whether it is based on mutation or initial heterogeneity and selection remains to be solved.

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